Molecular Basis for Recognition of Nucleoside Triphosphate by Gene 4 Helicase of Bacteriophage T7*

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The translocation of DNA helicases on single-stranded DNA and the unwinding of double-stranded DNA are fueled by the hydrolysis of nucleoside triphosphates (NTP). Although most helicases use ATP in these processes, the DNA helicase encoded by gene 4 of bacteriophage T7 uses dTTP most efficiently. To identify the structural requirements of the NTP, we determined the efficiency of DNA unwinding by T7 helicase using a variety of NTPs and their analogs. The 5-methyl group of thymine was critical for the efficient unwinding of DNA, although the presence of a 3′-ribosyl hydroxyl group partially overcame this requirement. The NTP-binding pocket of the protein was examined by randomly substituting amino acids for several amino acid residues (Thr-320, Arg-504, Tyr-535, and Leu-542) that the crystal structure suggests interact with the nucleotide. Although positions 320 and 542 required aliphatic residues of the appropriate size, an aromatic side chain was necessary at position 535 to stabilize NTP for efficient unwinding. A basic side chain of residue 504 was essential to interact with the 4-carbonyl of the thymine base of dTTP. Replacement of this residue with a small aliphatic residue allowed the accommodation of other NTPs, resulting in the preferential use of dATP and the use of dCTP, a nucleotide not normally used. Results from this study suggest that the NTP must be stabilized by specific interactions within the NTP-binding site of the protein to achieve efficient hydrolysis. These interactions dictate NTP specificity.

Helicases are ubiquitous enzymes that play pivotal roles in diverse cellular activities (1). Reactions mediated by helicases require their ability to bind and move along a DNA or RNA strand. Such a fundamental feature is coupled to the binding of nucleoside triphosphates (NTPs)[2] and their hydrolysis. An underlying mechanism in helicase action is a change in contact of the protein with the nucleic acid strand depending on the state of the bound NTP (1). Binding and subsequent hydrolysis of NTP induce conformational shifts in the nucleic acid-interacting part of the helicase that enable the enzyme to move unidirectionally. The unidirectional movement enables the helicase to translocate on the strand to which it is bound, and upon encountering duplex DNA, its continued movement results in unwinding of the dsDNA. Consequently, despite the divergence in their structure and mechanism of action, all helicases require the binding and hydrolysis of NTP to fulfill these multiple roles (2). Accordingly, signature structural motifs of helicase for NTP binding, for example the Walker motifs A and B, are conserved throughout the helicase family (3). These motifs interact with the phosphate moiety of NTP and influence DNA binding upon hydrolysis of NTP. In the case of hexameric helicases such as the T7 DNA helicase, complexity of helicase action is increased because the oligomerization of the subunits is a prerequisite for all the steps described above. In helicases of this family, binding and hydrolysis of NTP occur at the interface of two subunits (4, 5). A recent structural study of an AAA$^+$ hexameric helicase demonstrated that indeed a polypeptide hairpin of the helicase makes different contacts with the ssDNA depending on the hydrolysis state of the NTP (6).

The helicase responsible for unwinding dsDNA to provide an ssDNA template for the replicative DNA polymerase of bacteriophage T7 is encoded by gene 4 of the phage. The multifunctional gene 4 protein contains a helicase domain located in the C-terminal half of the protein and a primase domain located in the N-terminal half. The helicase domain, along with the linker connecting the helicase and primase domains, contains the determinants for hexamer formation (7). The T7 gene 4 helicase, like other homologs in the DnaB-like family (Family 4), functions as an oligomer (4, 8, 9). The unique coexistence of both primase and helicase domains in a single polypeptide bypasses the requirement for assembly of the two proteins as it must occur in other replication systems. In addition, the primase benefits from the relatively tight binding of the helicase to DNA, and its location in the N-terminal half of the protein places it in the proper position to survey the DNA exiting from the translocating helicase for primase recognition sites (10).

Another feature unique to the T7 gene 4 helicase is its preference for dTTP, whereas most other helicases prefer ATP, the most abundant NTP in the cell (11), in the unwinding reaction (12–15). Some helicases can use dATP, GTP, or dCTP (13, 16, 17) almost as efficiently as ATP. However, preferential use of dTTP over ATP has not been observed by any helicase other than the T7 helicase. Preferential use of dTTP by T7 gene 4 protein was noticed initially in studies on strand displacement DNA synthesis mediated by T7 DNA polymerase and T7 DNA helicase (18). In this reaction, the helicase unwinds the duplex DNA to generate ssDNA template for the DNA polymerase. Measurement of nucleoside diphosphate arising during DNA synthesis revealed that the T7 helicase most favorably uses

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[2] The abbreviations used are: NTP, nucleoside triphosphate; ssDNA, single-stranded DNA; Ni-NTA, nickel-nitrilotriacetic acid; rNTP, ribonucleotide triphosphate; 4S-dT, 4-thiothymidine 5′-triphosphate; 5-Me-dC, 5-methyl-2′-deoxycytidine 5′-triphosphate; 5-Br-dU, 5-bromo-2′-deoxyuridine 5′-triphosphate; 5-Pr-dU, 5-propynyl-2′-deoxyuridine 5′-triphosphate.
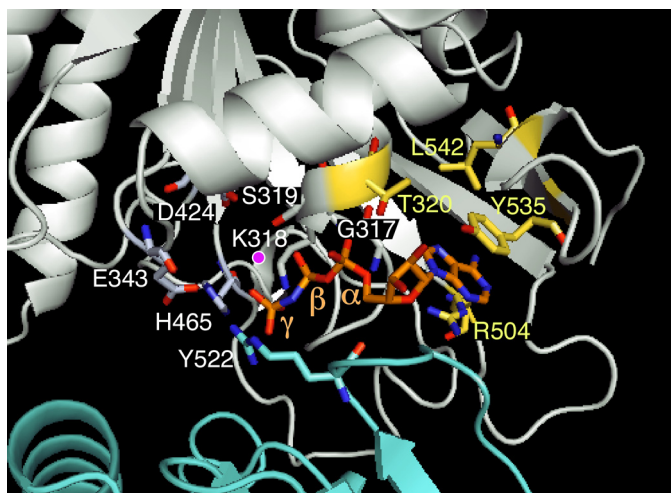
FIGURE 1. **NTP-binding pocket of T7 gene 4 helicase.** The X-ray crystallographic structure of the T7 DNA helicase (Protein Data Bank code 1E0J) (9) reveals the NTP-binding site located at the interface of the two subunits shown in *white* and *cyan*. The non-hydrolyzable analog of ATP (β,γ-imido ATP) shown in *orange* with the α-, β-, and γ-phosphates is cradled inside the binding pocket. Residues close to the phosphates (Gly-317, Lys-318, Ser-319, Glu-343, Asp-424, His-465, and Tyr-522) and thus critical to NTP hydrolysis are indicated. A magnesium ion found in the binding site is shown as a *magenta dot*. The four residues (Thr-320, Arg-504, Tyr-535, and Leu-542) selected in this study for site-directed mutation are indicated in *yellow*. They are in close proximity to the base and/or sugar of the ATP analog.

dTTP and dATP followed by ATP (18). Subsequent studies have shown that indeed either dTTP or dATP can support the unwinding of dsDNA by the T7 helicase (19). However, the hydrolysis of dTTP by T7 DNA helicase is not limited to the unwinding of dsDNA as the hydrolysis of dTTP is also required for the unidirectional translocation of the helicase on ssDNA. In this reaction, dTTP is also preferred, although all NTPs except CTP are hydrolyzed (20). The K_m for dTTP in this reaction is 0.4 mM, whereas that for ATP is an order of magnitude higher (3.2 mM). The K_m for dATP, the NTP that is also used favorably, is 1.2 mM. The use of dNTP by the helicase for translocation is complex in that the functional helicase is a hexamer with six NTP-binding sites, each of which is capable of hydrolyzing NTP in a random manner (21). Consequently, the occupancy of NTP-binding sites other than the one fueling translocation of the DNA may have an effect on translocation on the DNA. Recent studies using genetically modified helicases suggest that T7 helicase uses the energy derived from the hydrolysis of dATP in addition to dTTP for mediating unwinding of dsDNA (19).

X-ray crystallographic structures of the T7 helicase in complex with various nucleotides provide molecular details on the NTP-binding pockets (4, 9). The NTP-binding site located at the interface of two subunits within the hexameric helicase is composed of several conserved residues (Fig. 1). Essential roles of many of these residues have been demonstrated by site-directed mutagenesis followed by biochemical characterization of the altered proteins (21–26). These roles include sensing the state of hydrolysis during catalysis of NTP hydrolysis (Gly-317 and Lys-318), coordination of metal ions (Ser-319 and Asp-424), providing the catalytic base (Glu-343), and communication between the bound DNA and the NTP (His-465). In addition, the arginine finger, Arg-522, of the subunit adjacent to the

NTP contacts the γ-phosphate of NTP to couple NTP hydrolysis to conformational changes in the subunit. Although the residues mentioned above are located in proximity to phosphate groups of the NTP, structural studies also demonstrate that the base of the NTP is stacked between the side chains of two residues, Tyr-535 and Arg-504 (9), implicating a role for these residues in NTP binding.

Precisely which structural features of the NTP-binding pocket dictate specificity for dTTP remains unclear. A recent study (19) revealed that Arg-363, in close proximity to the sugar of the bound NTP, is essential for the hydrolysis of dATP, dCTP, and dGTP. In this study, Arg-504 and Ser-319 were found to be important in the hydrolysis of dTTP. For example, substitution of alanine for Arg-504 increases the rate of hydrolysis of dCTP and enables this nucleotide to fuel unwinding of DNA, and substitution of threonine for Ser-319 reduces the hydrolysis of dTTP without affecting that of dATP. Interestingly, bacterial helicases have a threonine at the position corresponding to Ser-319 in the T7 helicase and preferentially use ATP. In the current study, we examined the ability of a number of NTPs and their analogs to replace dTTP in the unwinding reaction in an attempt to identify the structural features of the NTP that are important for activity of the helicase. Based on structural information of the T7 helicase (4, 8, 9), we selected four residues within the NTP-binding site that have the potential to play a role in NTP specificity. Rather than selectively substitute an amino acid for each of the four found in the helicase, we randomly replaced the four residues and examined the consequences of these replacements. By examining NTP analogs and site-directed mutagenesis in the NTP-binding site of T7 helicase, we identified specific interactions between the NTP and side chains of residues in the binding pocket that stabilize the NTP and support efficient translocation on ssDNA and unwinding of dsDNA.

EXPERIMENTAL PROCEDURES

Materials

Primers used for site-directed mutagenesis were obtained from Integrated DNA Technology. DNA purification kits, Ni-NTA resin, and Ni-NTA minispin columns were purchased from Qiagen. Restriction endonucleases, T4 DNA ligase, and Deep Vent® DNA polymerase were from New England Biolabs. Slide-A-Lyzer® minidialysis units were from Pierce. NTP analogs were purchased from TriLink Biotechnologies.

Methods

Construction of Gene 4 Protein Library—A library of gene 4 proteins containing a single amino acid substitution at specific positions was generated as follows. DNA fragments containing random mutations were generated by standard PCR using a mutagenic primer harboring mixed codon NNK (N = any base, K = G or T) at the designated position (27). The DNA fragments were digested with restriction endonucleases and ligated into a plasmid encoding the His-tagged gene 4 protein (pET28-gp4) previously cut with the same restriction enzymes. After transformation of the ligation products, resulting plasmids were isolated from individual colonies, and the entire gene 4 coding region of each plasmid was verified by DNA sequence

analysis. To generate mutations containing two separate single amino acid substitutions, PCR was carried out as described above using a plasmid containing a single mutation as template along with mutagenic primers containing another single mutation.

Complementation Assay for Growth of T7 Gene 4 Deletion Phage—*Escherichia coli* DH5α containing a plasmid encoding T7 gene 4 was grown to an A_{600} of ~1. About 0.1 ml of the bacterial culture was mixed with 3 ml of LB medium containing 0.7% agar and plated on an LB plate containing kanamycin. A small amount (1–2 μl) of serially diluted phage solution was spotted on the plate and incubated at 37 °C overnight.

Purification of His-tagged Gene 4 Proteins—Proteins used for the initial biochemical screening were prepared by one-step affinity purification using a Ni-NTA minispin column. Gene 4 protein containing a His$_6$ tag at the N terminus was overproduced in *E. coli* BL21(DE3) by inducing with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 3 h at an A_{600} of 1. The bacterial cells from a 100-ml culture were harvested, resuspended in 2 ml of buffer A (20 mM Tris-HCl, pH 7.5, and 0.1 M NaCl), and freeze-thawed three times in the presence of lysozyme (0.2 mg/ml) to rupture the cells. Clear bacterial lysate was collected by centrifugation and loaded onto the Ni-NTA spin column. After spinning, the column was washed with 5 ml of buffer A containing 0.1 M imidazole, and the protein was eluted with 0.3 ml of buffer A containing 0.5 M imidazole followed by dialysis against storage buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 50% glycerol). Protein purity was greater than 70% as judged by SDS-PAGE analysis followed by Coomassie staining. Large scale purification of gene 4 protein was carried out as described previously (28).

DNA Unwinding Assay—A minireplication fork consisting of a 65-mer and a 5′-^{32}P-radiolabeled 45-mer DNA was used as substrate for the DNA unwinding assay (29). The partial duplex substrate was further purified on a 10% native polyacrylamide gel to remove remaining ssDNA that might provide a substrate for the translocation activity of gene 4 helicase. A standard reaction mixture contained 100 nM DNA substrate, 1 mM indicated NTP, and 17 nM gene 4 protein (hexameric concentration) in reaction buffer B (40 mM Tris-HCl, pH 7.5, 50 mM potassium glutamate, 10 mM MgCl$_2$, and 10 mM DTT). The unwinding reaction was carried out at 37 °C for 5 min and terminated by addition of EDTA to a final concentration of 10 mM. After the reaction products were separated on a 10% native gel, the amount of radioactivity in the displaced 45-mer was measured using a Fuji BAS 1000 BioImaging analyzer. All data were from at least two independent experiments.

NTP Hydrolysis Assay—A range of gene 4 protein concentrations (from 10 to 600 nM) was incubated with 100 μM indicated NTP (0.25 μCi of [α-^{32}P]NTP) in reaction buffer B. The reaction mixture was incubated at 37 °C for either 60 min in the absence of DNA or 20 min in the presence of 9 nM M13 ssDNA. The reaction was terminated by adding EDTA to a final concentration of 25 mM. Reaction products were separated by polyethyleneimine cellulose thin layer chromatography (28) and analyzed by a Fuji BAS 1000 BioImaging analyzer.
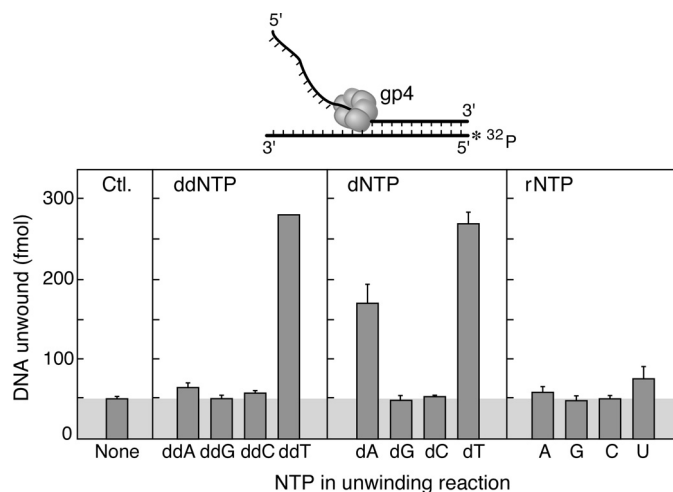

FIGURE 2. **Ability of NTPs to support unwinding of dsDNA by T7 gene 4 helicase.** Unwinding activity of T7 helicase was determined by measuring displacement of ssDNA from a dsDNA substrate. The DNA substrate (*top*) consists of a 65-mer partially annealed to a 5′-^{32}P-labeled 45-mer. The 65-mer has a 40-nucleotide 5′-ssDNA tail to which the helicase can assemble and 20 nucleotides at the 3′-end of the 45-mer that are not base-paired. The reaction mixture contains 100 nM DNA, 1 mM indicated NTP, and 17 nM T7 gene 4 protein (hexameric concentration). After incubation at 37 °C for 5 min, the products of the reaction were separated on a 10% native polyacrylamide gel, and the amount of displaced radiolabeled 45-mer was determined by phosphorimaging analysis. The amount of DNA unwound by T7 helicase in the presence of the indicated NTP is shown in the bar graph. *Shaded* portions in *bars* indicate the background when NTP was omitted from the reaction *as shown in the control (Ctl.)* on the left. Error bars were derived from two independent experiments.

RESULTS

Use of NTPs and Their Analogs in Unwinding of dsDNA by T7 Helicase

Unwinding of dsDNA by T7 gene 4 helicase can be measured directly by observing the release of a radioactively labeled oligonucleotide partially annealed to a complementary oligonucleotide (29). The minireplication fork (Fig. 2, *inset*) has a 40-nucleotide 5′-ssDNA tail to which the helicase can assemble and a 20-nucleotide 3′-ssDNA tail. Because the unwinding reaction requires hydrolysis of NTP, the level of unwinding in the presence of NTP provides information as to how efficiently T7 gene 4 helicase uses a particular NTP. We measured the ability of various NTPs to support activity of T7 DNA helicase to unwind DNA (Fig. 2). In the assay, the minireplication fork was present in a 6-fold molar excess over the hexameric helicase to ensure that turnover of the helicase can occur in the reaction. In the initial experiment, we determined apparent K_m and V_{max} for the most frequently used NTPs, dTTP and dATP (Table 1). Although dTTP was preferentially used for unwinding (more than 3-fold higher V_{max} than dATP), both NTPs showed similar K_m values lower than 0.3 mM. In addition, the average cellular concentration of rNTPs is in the millimolar range (11). Therefore, we used 1 mM NTP for comparison with dTTP in unwinding assay. Overall, the preference for dNTPs in the unwinding reaction is in agreement with the previous observations measuring their efficiency in the coupled helicase-polymerase assay and in their hydrolysis during translocation on ssDNA (18, 20). The most efficient dNTP to support unwinding by the T7 helicase was dTTP followed by dATP; as found previously (19), dGTP and dCTP were essentially unable to support unwinding of DNA by the helicase. ddTTP was sim-

ilar to dTTP in supporting unwinding of DNA, but surprisingly ddATP was unable to do so. rNTPs were poor substrates for T7 DNA helicase with only UTP providing any detectable activity. Clearly the NTP-binding site of T7 helicase is optimized for dTTP with the unexplained ability to use dATP.

To identify structural components of dTTP contributing to the preference for DNA unwinding, we examined a variety of NTPs structurally related to dTTP for their ability to support unwinding activity. All NTPs containing thymine showed a significant level of unwinding regardless of the presence or location of the hydroxyl group on the sugar (Fig. 3*A*). When the base was replaced with

uracil, eliminating the 5-methyl group, only the configuration of 2′-dUTP (3′-OH, 2′-H) provided activity approaching that observed with dTTP. The presence of a 2′-OH (rUTP) rendered the NTP almost 7-fold less active. Collectively, these results suggest that the 5-methyl in the base is required to maintain an important contact in the binding pocket of T7 helicase. The absence of the 5-methyl in the base can be compensated by the absence of a 2′-OH and the presence of a 3′-OH.

Because modifications in the base of dTTP significantly affected its use in unwinding by T7 helicase, we further examined the effect of substitutions at positions 4 and 5 of the base on the unwinding activity. Although replacement of the carbonyl group at position 4 with a thio group (4S-dT) slightly reduced unwinding efficiency, substitution of an amino group (5-Me-dC) eliminated the activity (Fig. 3*B*), suggesting a critical interaction mediated by the carbonyl group. Similarly, the methyl group at position 5 could be replaced with a bromo group (5-Br-dU) of an equivalent size without affecting unwinding, but a bulky propynyl group (5-Pr-dU) at this position abolished activity to support unwinding.

Role of Amino Acid Residues in NTP-binding Pocket for Unwinding Activity

The NTP use by T7 gene 4 helicase presented above suggests that residues within the NTP-binding pocket of the protein are important in accommodating the 4-carbonyl, 5-methyl, and the 3′-OH groups of dTTP. Fortunately, crystal structures of the T7 helicase domain in complex with dTTP (Protein Data Bank code 1CR4) and a non-hydrolyzable ATP analog (Protein Data Bank code 1E0J) are available (4, 9). Based on these structures, we chose four residues located in proximity (4–5 Å) to the base and sugar of the dTTP for further examination. These residues (Thr-320, Arg-504, Tyr-535, and Leu-542) and their properties are listed in Table 2. Each of the selected residues was randomly replaced with other amino acids using a mixed codon (NNK where N = any base and K = G or T) at the selected position as described under "Experimental Procedures." Although this random mutagenesis significantly reduces redundancy to code all 20 possible amino acids (27), we were able to obtain only 9–14 different substitutions for the four positions after examination of ~90 clones for each position. It appears that statistically larger numbers of clones need to be examined to obtain all possible 19 substitutions.

TABLE 1

Apparent kinetic constants for NTP used by T7 gene 4 helicase in DNA unwinding reaction

The rate of unwinding of dsDNA by T7 helicase was measured as described under "Experimental Procedures" with a wide range of NTP concentrations (0–2.5 mM). K_m and V_{max} were calculated by a non-linear regression method using Enzyme Kinetics software (Trinity Software). Data were collected from at least three independent experiments. Because the unwinding assay measures complete separation of 25 bp in DNA substrate, data reported here do not represent the initial reaction rate for unwinding. Nonetheless, the apparent values are sufficient to determine the specificity for NTP use in the unwinding reaction.

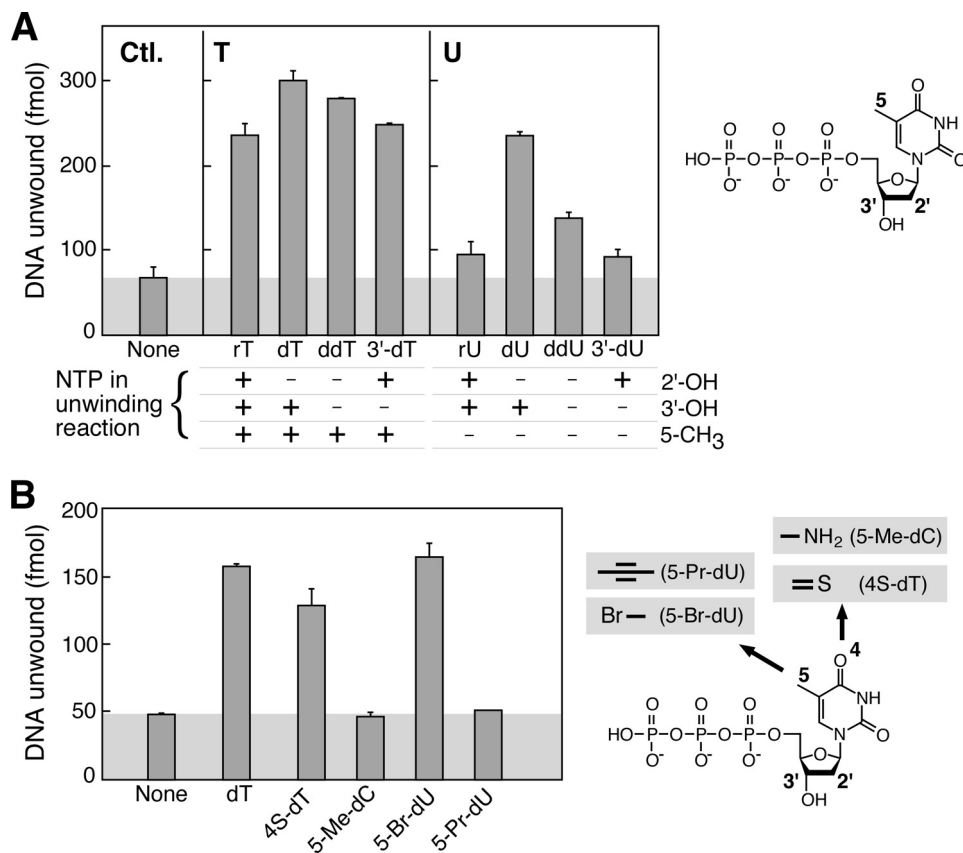|	K_m	V_{max}
	mM	*fmol dsDNA unwound/83 fmol hexameric helicase/5 min*
dTTP	0.22 ± 0.2	165 ± 11
dATP	0.24 ± 0.2	49 ± 8

FIGURE 3. Effect of modifications in base and sugar of dTTP on DNA unwinding. DNA unwinding reactions were carried out as described in the legend to Fig. 2 with 1 mM indicated NTP. *A*, modification in the 5-methyl of the base and hydroxyl groups of the sugar. The chemical structure of dTTP is shown at the *right*. The presence of a 2′- and/or 3′-hydroxyl group on the sugar and 5-methyl group on the base is noted *below* each NTP examined. *B*, modification in position 4 or 5 of the base. Chemical structures of the group substituted for the 4-carbonyl or 5-methyl are shown. *Ctl.*, control. *Error bars* were derived from two independent experiments.

TABLE 2

Residues of T7 helicase randomly substituted

Four residues whose side chains are in proximity to the base and sugar of co-crystallized NTP were selected. In the structure of T7 helicase complexed with non-hydrolyzable ATP (Protein Data Bank code 1E0J), the side chains are located within 4–5 Å to various parts of the ATP analog.

Residue	In proximity to	Secondary structure	Conserved in Family 4 helicase
Thr-320	Sugar	α helix	Thr/Ala/Ser/Leu
Arg-504	Base	Loop	Arg
Tyr-535	Sugar, base	β strand	Tyr/Phe
Leu-542	Base	β strand	Various

In total, 47 altered helicases containing a single amino acid substitution were overproduced. The proteins were purified as a fusion protein containing six histidine residues at the N terminus using Ni-NTA affinity column chromatography. The presence of the histidine tag did not affect protein function as judged by phage complementation assay and DNA unwinding assays (data not shown). Unwinding activity of the altered helicases was examined in the presence of specific NTP(s) (Fig. 4). As the wild-type helicase did not efficiently use NTPs other than dTTP and dATP, we used mixtures of the less used NTPs
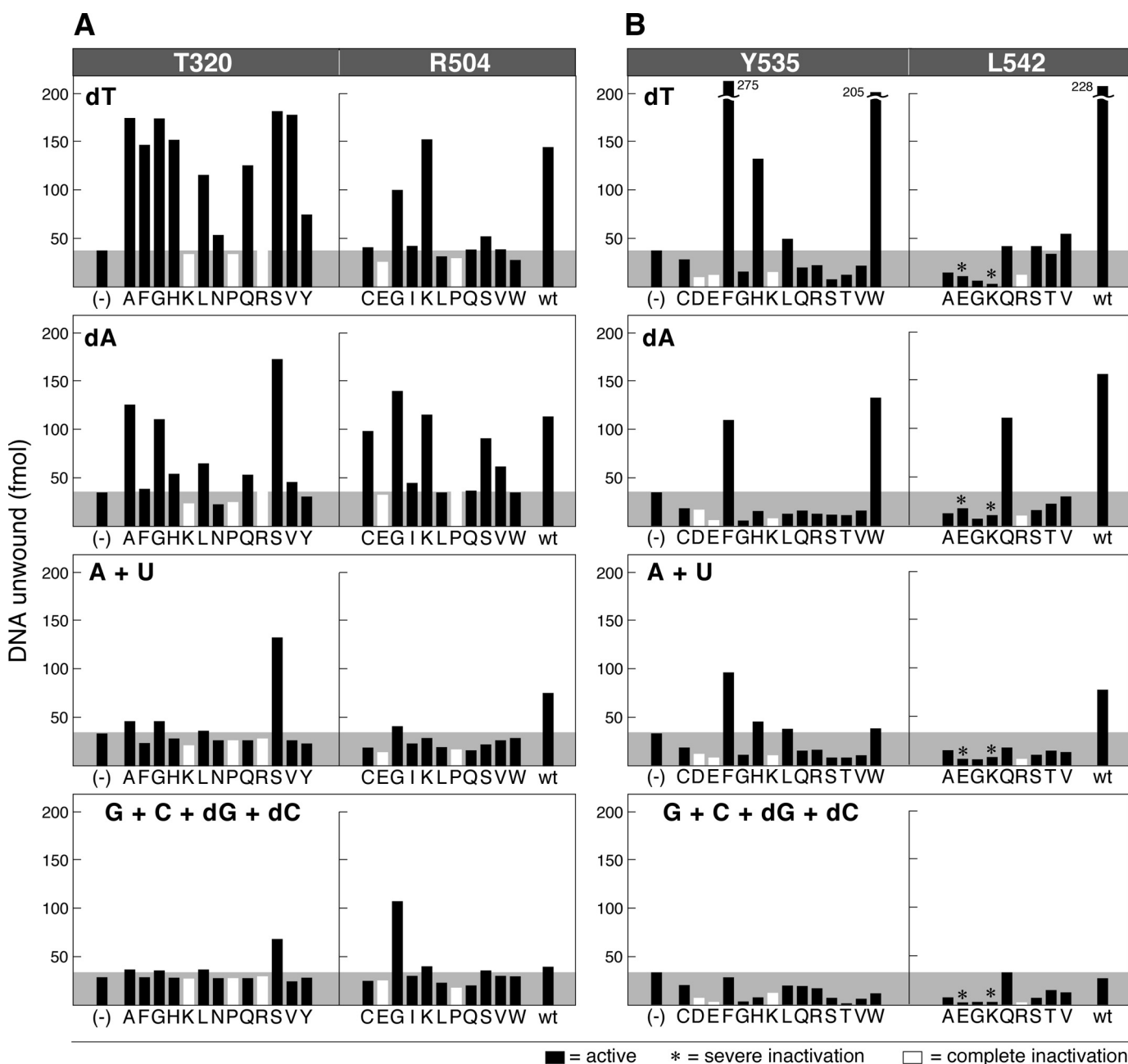

FIGURE 4. **Use of NTPs by altered gene 4 proteins containing single amino acid substitution.** Altered gene 4 proteins were purified using Ni-NTA affinity column chromatography. Unwinding assays were carried out in the presence of the indicated NTP(s). In *A* and *B*, the amino acid substituted for the original amino acid in the helicase is indicated on the *x axis*, and the unwinding activity catalyzed by each altered protein is presented in a bar graph. *Unshaded bars* indicate that the alteration in the helicase leads to an inability to complement T7Δ4 phage lacking gene 4 (at least a 1000-fold reduction in efficiency of plating compared with wild-type gene 4). *Bars* with an *asterisk* denote that the alteration did not result in a significant reduction in plating efficiency but did result in plaques smaller than those produced with wild-type protein. (−) and *wt* indicate that no protein and the wild-type gene 4 protein were present in the reaction, respectively. Standard error bars are omitted for clarity.

in the initial screening. The results of the screening assay for each of the four residues mutated are presented below.

Threonine 320—Substitutions of non-charged residues for this position gave rise to helicases that exhibit an NTP use profile similar to that of wild-type helicase. Particularly, replacement with a small aliphatic residue (Ala or Gly) demonstrated almost no change from the wild-type protein. It is noteworthy that gp4-T320S uses dATP as efficiently as it does dTTP and also uses nucleotides that are poor substrates for the wild-type enzyme as evidenced by its use of the A + U mixture and the G + C + dG + dC mixture. The only exception to NTP use unaffected by replacement with a neutral residue was observed with gp4-T320P. This altered protein could not use any NTP and thus could not support growth of T7 phage lacking gene 4 (T7Δ4). Likewise, replacement with a basic residue (Lys or Arg) also abrogated the ability to use any nucleotide for unwinding and to complement T7Δ4 phage. These results suggest the requirement for a hydrophobic environment at this position. The remainder of the substitutions produced proteins whose unwinding activities were more or less similar to that of wild-type protein in the presence of dTTP. However, these altered helicases did not use NTPs other than dTTP as efficiently as did the wild-type protein.

Arginine 504—Replacement of Arg-504 with a small aliphatic residue (Cys, Gly, Ser, or Val) led to a preference of dATP over dTTP. However, this shift in NTP use was not observed with larger aliphatic residues (Ile and Leu). Most striking is the finding that the R504G alteration gave rise to a helicase that catalyzed unwinding with the mixture of CTP, dCTP, GTP, and dGTP. Wild-type gene 4 protein could not use this nucleotide mixture to catalyze unwinding of DNA. gp4-R504C used dATP but no longer used dTTP. On the other hand, replacement with lysine revealed a pattern very similar to that of the wild-type enzyme, suggesting the importance of a basic residue at this position. A change to proline or acidic glutamic acid resulted in a loss of ability to complement T7Δ4 phage and a loss of activity with all NTPs.

Because the alteration in gp4-R504G revealed one of the most drastic changes in NTP use, we purified the altered protein and examined its nucleotide specificity (Fig. 5). gp4-R504G has several features of NTP use different from that of wild-type gene 4 protein. Most significantly, the altered protein no longer preferred dTTP but instead preferred dATP and could also use dCTP. The latter nucleotide could not be used by wild-type helicase to unwind DNA. Thus, this versatile use of NTPs enables gp4-R504G to use any dNTP except dGTP for unwinding of DNA. gp4-R504G could also unwind DNA, albeit to a lesser extent, using ddCTP and ddATP. In a separate study, we found that substitution of alanine for Arg-504 also led to a preferred use of dCTP (19). We further measured the time course of DNA unwinding in the presence of the most efficiently used dNTP (dTTP for the wild-type and dATP for gp4-R504G). The results show that the altered protein unwound DNA somewhat faster than did the wild-type T7 helicase (Fig. 6). We also examined dTTP analogs that contain bulky groups at position 4 or 5 of the base (5-methyl dCTP or 5-propynyl dUTP) and thus could not be used by wild-type helicase (Fig. 3*B*). Both analogs were used as well as dTTP by gp4-R504G (data not shown).
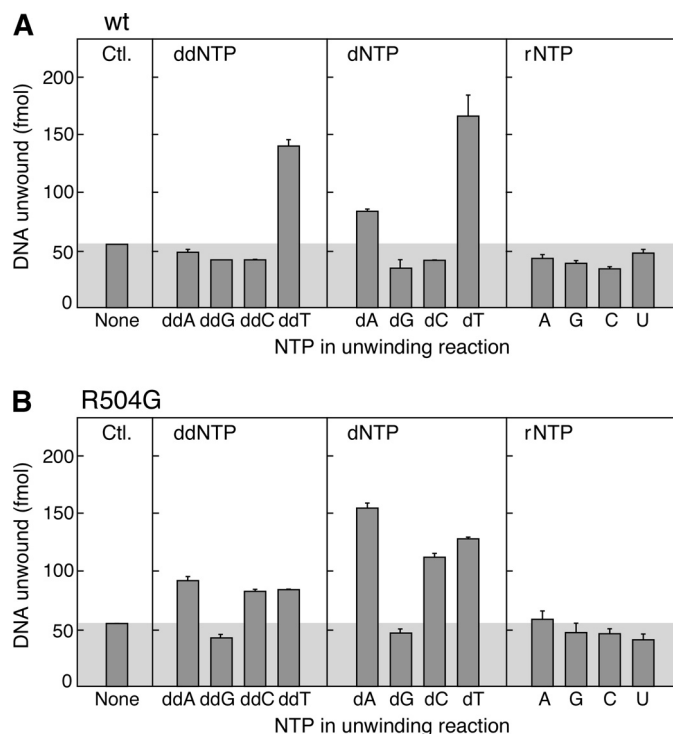
FIGURE 5. **Use of NTPs by gp4-R504G.** Unwinding efficiency of either wild-type T7 helicase (*A*) or gp4-R504G (*B*) was measured in the presence of the indicated NTPs. *Ctl.*, control. *Error bars* were derived from two independent experiments.
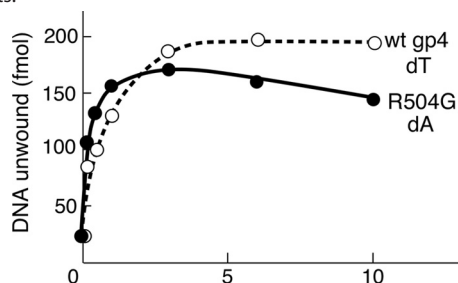
FIGURE 6. **Time course of unwinding by wild-type T7 helicase or gp4-R504G.** Unwinding catalyzed by wild-type protein or gp4-R504G in the presence of dTTP or dATP, respectively, was determined at the indicated times. Representative data from multiple experiments are shown.

Binding and hydrolysis of NTP are a prerequisite for DNA unwinding by the T7 helicase. To further investigate the NTP-binding pocket of T7 helicase, hydrolysis of NTPs in the presence or absence of ssDNA was examined (Table 3). T7 gene 4 helicase hydrolyzed NTPs in the absence of DNA at a rate considerably less than that observed in the presence of ssDNA (30). Wild-type DNA helicase hydrolyzed dTTP and dATP at almost identical rates in the absence of DNA (Table 3). Not surprisingly, dCTP, a nucleotide not used for unwinding, was hydrolyzed at a very low rate. Replacement of Arg-504 with glycine reduced the hydrolysis of dTTP and dATP to the same low level observed with dCTP.

In the presence of ssDNA, dTTP was hydrolyzed about 3-fold better than dATP by the wild-type helicase, suggesting that DNA binding induces conformational changes in the NTP-binding pocket more suitable for dTTP than dATP. In the case of gp4-R504G, the rate of hydrolysis was comparable for all the NTPs with some preference for dATP. Compared with wild-

TABLE 3

Hydrolysis of dNTP by T7 helicases

In each experiment, the rate of hydrolysis of the NTP was proportional to the concentration of gene 4 protein. From the linear range, the rate of NTP hydrolysis was determined by dividing the amount of NTP hydrolyzed per hour by the amount of T7 helicase (pmol of nucleoside diphosphate (NDP)/fmol of helicase/h). Reaction mixtures were incubated at 37 °C for either 60 min in the absence of DNA or 20 min in the presence of DNA.

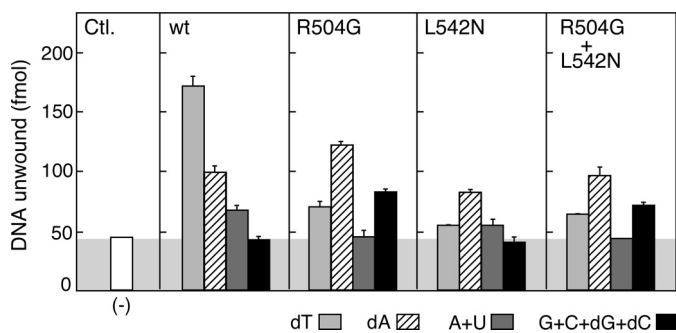|	In the absence of DNA			In the presence of DNA		
	dTTP	dATP	dCTP	dTTP	dATP	dCTP
	pmol NDP/fmol of helicase/h			*pmol NDP/fmol of helicase/h*		
gp4-wt	0.33 ± 0.01	0.32 ± 0.04	0.05 ± 0.002	3.49 ± 0.65	1.13 ± 0.02	0.14 ± 0.01
gp4-R504G	0.02 ± 0.001	0.04 ± 0.006	0.02 ± 0.003	0.93 ± 0.07	1.56 ± 0.08	0.80 ± 0.04

FIGURE 7. **Use of NTPs by gp4-R504G/L542N.** The altered gene 4 protein containing two amino substitutions (gp4-R504G/L542N), each of which alone increased the specificity for dATP, was purified using Ni-NTA affinity column chromatography. An unwinding assay was carried out in the presence of the indicated NTP. (−) indicates that no protein was present in the reaction. *Ctl.,* control. *Error bars* were derived from two independent experiments.

type protein, the substitution for Arg-504 increased use of dCTP more than 5-fold but decreased the use of dTTP by 3.5-fold. No significant change in dATP use by the substitution was observed. A similar level of hydrolysis for all the NTPs by the altered protein insinuates that Arg-504 is again an important determinant for NTP selectivity after ssDNA is bound. Overall, the NTP preference observed from NTP hydrolysis kinetics in the presence of DNA (Table 3), which might reflect translocation on ssDNA, was consistent with the NTP preference observed in the DNA unwinding studies (Table 1 and Fig. 5).

Tyrosine 535—Only replacement of Tyr-535 with a residue containing an aromatic side chain (Phe or Trp) provided for significant catalytic activity, clearly demonstrating a requirement for an aromatic group at this position. In particular, phenylalanine could be substituted for tyrosine without affecting activity. Whereas changes to any charged residue (Asp, Glu, or Lys) were detrimental to the protein function, replacement with histidine resulted in diminished but significant use of dTTP, another indication for a requirement for an aromatic ring at this position. Although the rest of the altered helicases did not show obvious unwinding activity with any NTPs, they were able to complement T7Δ4 phage. This discrepancy between *in vivo* and *in vitro* data is often observed and may be explained by the reduced helicase activity of the altered proteins being compensated by overproduction of the protein under the *in vivo* complementation assay condition.

Leucine 542—Most alterations at this position significantly reduced unwinding activity in the presence of dTTP. Only gp4-L542Q exhibited significant unwinding activity and only in the presence of dATP. As described below, we also examined gp4-L542N and found that a change to either glutamine or asparagine resulted in the same pattern for NTP use (Fig. 7). A

decrease in the size of the side chain (Ala or Gly) resulted in a reduction of unwinding activity with any NTPs. Replacements with a charged residue (Glu, Lys, or Arg) also significantly decreased the use of any NTP. Although gp4-L542E and gp4-L542K supported growth of T7Δ4 phage at a reduced level as indicated by small plaques, gp4-L542R did not complement T7Δ4.

Not surprisingly, altered proteins that cannot complement T7Δ4 phage did not catalyze unwinding of DNA with any NTP. However, none of the altered proteins exhibited a dominant negative effect on the wild-type helicase expressed by T7 phage (data not shown). Primase activity of the altered gene 4 proteins was measured by primer synthesis assay. No difference from the wild-type protein was found with any of the proteins, suggesting that alterations by the single amino acid substitutions were confined within the helicase domain (data not shown).

Effect of Combining Amino Acid Substitutions on NTP Use

From the above studies, several alterations of single amino acid substitutions that result in NTP use distinct from that of the wild-type T7 helicase were identified. Among those, the most frequently observed change in NTP use was a preference for dATP over dTTP. Alterations at two different positions, residues 504 and 542, both resulted in this preference of the helicase for dATP. In an attempt to augment the effect of the changed use pattern, we constructed a T7 helicase that contained both the R504G and L542N alterations. The resulting gp4-R504G/L542N still complemented T7Δ4 for growth. The altered protein containing both mutations showed an NTP use pattern intermediate between gp4-R504G and gp4-L542N (Fig. 7). The specificities conferred by each alteration were not additive.

DISCUSSION

DNA unwinding by helicase is a complex process in which binding of DNA and binding and hydrolysis of NTP are closely interrelated (31). In DnaB-like hexameric helicases, binding of an NTP at the interface of neighboring subunits enhances formation of the functional oligomeric helicase and, in turn, increases the affinity of DNA. Further hydrolysis of the NTP leads to conformational changes in the protein that move the DNA unidirectionally through the central channel of the helicase. Central to dissecting these processes is an understanding of the binding of the NTP within the nucleotide-binding pocket and the structural determinants of nucleotide specificity. In the current study, we examined the important structural aspects of the NTP itself and further extended our studies on the identification of residues within the nucleotide binding site that are

important to binding and hydrolysis and in transferring the state of hydrolysis of the nucleotide to unwinding of DNA.

Role of Residues in NTP-binding Site of T7 Helicase—The replacement of selected amino acid residues within the nucleotide-binding site with random residues provides the maximal possibility of identifying critical residues. Among the residues examined, the most tolerable site to amino acid changes was position 320. Any residues with a small aliphatic side chain at this position were sufficient for supporting unwinding of DNA, suggesting that no significant charge-mediated interaction is involved at this position. Both positions 320 and 542 required aliphatic residues of the appropriate size. Position 542 required a relatively large aliphatic side chain, presumably to interact with the 5-methyl group of thymine (4), a critical structural component in dTTP for DNA unwinding. Smaller aliphatic or charged residues at this position could not support use of any NTP.

The side chain of Tyr-535 makes extensive contacts with both the base and sugar of the NTP, positioning the aromatic ring parallel to the base (4, 9). The necessity of the planar side chain at this position, suggested from the structural studies, was confirmed in this investigation. An amino acid sequence alignment of DnaB-like helicases shows that only T7 helicase has a tyrosine at this position, whereas most other homologs contain phenylalanine (4). Consequently, it was speculated that the tyrosyl hydroxyl group at this position of T7 helicase might play a role in the unique NTP specificity. However, the hydroxyl group does not appear to be involved in any specific interactions that would explain a preference for dTTP. The role of the aromatic side chain appears to be primarily in stabilizing the NTP through stacking with the base.

In an earlier study (19), we identified Arg-504 as an important residue in determining nucleotide specificity. For example, gp4-R504A hydrolyzed dCTP far better than wild-type helicase. We extended the examination of this residue by replacing it with random amino acids. We found that only basic (Arg or Lys) residues at position 504 provide preference for dTTP over dATP in unwinding by the helicase. Deletion of the basic side chain resulted in significant loss of dTTP use, suggesting a specific interaction with the thymine base. We postulate that the guanidino group in the side chain of Arg-504 hydrogen bonds with the 4-carbonyl group of thymine to stabilize the dTTP (4). Replacement of the carbonyl with a similar but somewhat bulkier thio group did not significantly change unwinding efficiency. However, as discussed below, substitution of an amino group abolished the ability to support unwinding. This position is particularly interesting because replacement of the arginine with glycine or alanine resulted in the use of dCTP for unwinding. The inability of wild-type T7 helicase to use dCTP, a pyrimidine triphosphate similar to dTTP, could be due to the lack of an essential hydrogen acceptor at position 4 in dCTP. The 4-amino group in dCTP would give rise to repulsion on the basic side chain of Arg-504. Elimination of the basic group as in gp4-R504G not only removes the repulsion but also provides a wider NTP-binding pocket that accommodates any NTPs except for dGTP. The faster rate of DNA unwinding found with gp4-R504G insinuates that the altered binding pocket has an easier access for NTPs than that of wild-type protein. This substitu-

tion in the binding pocket accommodated dTTP analogs containing a bulky group in position 4 or 5 at the base.

How can the binding pocket of T7 helicase effectively use two structurally dissimilar nucleotides, dTTP and dATP? Examination of DNA unwinding using a variety of dTTP analogs together with site-directed mutagenesis revealed specific interactions between dTTP and residues within the NTP-binding pocket. To accommodate dATP, a planar side chain at position 535 was necessary to stabilize the NTP by base stacking, but a simple rotation of the base would not be sufficient to accommodate the bulky purine base in dATP. Instead, dATP could bind with an orientation distinct from that of dTTP that would require novel interactions with residues surrounding the base. Although we did not specifically attempt to define interactions necessary for dATP use, several observations support a binding distinct from that of dTTP. For example, gp4-T320F and gp4-Y535H used dTTP in a manner similar to wild-type helicase, but the use of dATP was reduced considerably. In contrast, replacements of Arg-504 with small aliphatic residues did not significantly change the efficiency in use of dATP but did decrease the use of dTTP, suggesting that the basic side chain at this position is not involved in critical interaction with dATP. Elimination of the 3′-hydroxyl group from the sugar also resulted in different effects as seen in the use of ddTTP and ddATP.

Specific NTP Use in DNA Unwinding—As discussed above, many residues in the NTP-binding pocket of the helicase play roles in positioning NTP and its efficient hydrolysis during unwinding reaction. Although contacts with the phosphate groups of NTP occur at the P-loop in the binding site, the base of the NTP is stabilized largely by stacking interaction with Tyr-535 (4, 9). Specificity toward binding and hydrolysis of a particular NTP is determined by further interactions, exemplified best by hydrogen bonding between the guanidino side chain of Arg-504 and the 4-carbonyl group of the thymine base. In the absence of ssDNA, both NTPs were hydrolyzed at a similar rate, but binding of ssDNA increased the selectivity for dTTP (Table 3). We propose that the NTP-binding pocket is initially less selectively predisposed to certain NTPs, but ssDNA binding induces conformational changes that accommodate a particular NTP to align for stable binding and efficient hydrolysis. As a result, only selected NTPs can support translocation on ssDNA or unwinding of dsDNA.

Although we examined residues located close to the base or sugar of the NTP in this study, we also observed that alterations in residues located near the phosphates of NTP can result in different NTP use (19). Hence, it is likely that the overall binding of NTP in the binding pocket occurs in an induced fit manner with gradual adjustment of the NTP position through specific interactions with residues in the NTP-binding pocket.

NTP Binding of Other DnaB Family Helicases—T7 DNA helicase is a member of the family of hexameric helicases whose structures and biochemical activities are well characterized. A recent report on *Thermus aquaticus* (*Taq*) DnaB, another hexameric helicase, illustrates the conserved nature of the NTP-binding site in the homolog (32). Modeling of *Taq* helicase with non-hydrolyzable ATP in comparison with the T7 helicase structure shows that the NTP-binding pocket resembles that of

T7 helicase. Similar to the case of T7 helicase, side chains of the two residues (Arg-397 and Phe-430) corresponding to Arg-504 and Tyr-535 of T7 helicase sandwich the adenine base of the ATP analogs in *Taq* helicases (32).

Because the replacement of Arg-504 with glycine or alanine resulted in a drastic effect on the use of NTP by T7 helicase, we examined the role of the homologous residue in *E. coli* DnaB (Arg-420) by introducing the equivalent replacement of R420G. Comparison of the altered *E. coli* DnaB with the wild-type in NTP use for DNA unwinding revealed that both proteins commonly use only two NTPs: a preference for ATP and followed by dATP (data not shown). No significant change in nucleotide specificity was observed with the substitution of glycine for Arg-420, suggesting that the role of the conserved arginine in *E. coli* helicase is dissimilar to that in T7 DNA helicase.

Strategic Use of NTP by T7 Helicase—One motivation for this study was to address the unique specificity of T7 gene 4 helicase for dTTP in DNA unwinding. ATP is the most abundant NTP in the bacterial cell (11), yet T7 helicase uses dTTP, a nucleotide ~40-fold less abundant than ATP. As ATP is the most common nucleotide used by a wide range of enzymes, T7 helicase could avoid competition with many other enzymes that use ATP including the host helicase (*E. coli* DnaB) by using dTTP. In addition, considering that the primase domain in the same polypeptide uses ATP and CTP for primer synthesis, it would be more efficient to use other NTPs for the helicase activity to evade depletion of ATP. Indeed, recent studies indicate that changes in the pool of CTP may have drastic effects on primer synthesis and concomitantly DNA replication.[3] On the other hand, the use of dTTP, present in a limited amount, would give rise to more effective control of the overall NTP pool by T7 phage. A recent report on the role of T7 gene 1.7 protein (33) suggests that T7 phage can actively manipulate the NTP pool for its effective growth by modulating dNTP metabolism. The product of T7 gene 1.7 is a nucleotide kinase that specifically phosphorylates dTMP and dGMP to eventually yield dTTP and dGTP (33). The acquisition of this unique kinase could have arisen to assure an abundance of dTTP, or the helicase could have acquired specificity for dTTP as a consequence of its abundance due to the T7 kinase. In another scenario, depletion of a specific NTP might serve as a signal for subsequent processes during phage infection.

T7 DNA helicase uses dATP although not as efficiently as it does dTTP. The ability of the enzyme to use more than one NTP would be advantageous in competing with host enzymes for NTPs. As discussed above, the NTP-binding site of *E. coli* DnaB does not appear sufficiently flexible to accommodate NTPs other than ATP or dATP (34). Interestingly, helicases from many parasites can use multiple NTPs in the unwinding reaction (17, 35, 36). It is assumed that T7 helicase might use dTTP *in vivo* for its helicase activity because dTTP is the most effective NTP in most helicase assays examined *in vitro*. Recently, studies with genetically altered T7 DNA helicase in which residues in the NTP-binding site were altered suggest that nucleotides other than dTTP can increase the overall rate

of DNA unwinding where dTTP provides the energy for translocation (19). In this model, the five NTP-binding sites on the hexameric helicase other than the one providing the energy for translocation could be occupied by NTPs other than dTTP. Evidence suggests that dATP, as well as dTTP, may be required by the T7 helicase for DNA replication and phage growth (19).

In summary, we identified critical structural determinants in the NTP as well as the NTP-binding pocket of T7 helicase that confer specific use of NTPs by the helicase. The NTP-binding pocket of T7 helicase is suited for binding either dTTP or dATP, but upon binding ssDNA, the use of dTTP is favored for DNA unwinding. The unique NTP specificities of DNA helicases provide a reasonable target for drug therapy (37, 38).

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